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Molecular Analysis of Chromosome 21 in a Patient With a Phenotype of Down Syndrome and Apparently Normal Karyotype

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Down syndrome (DS) is caused in most cases by the presence of an extra chromosome 21. It has been shown that the DS phenotype is produced by duplication of only a small part of the long arm of chromosome 21, the 21q22 region, including and distal to locus D21S55. We present molecular investigations on a woman with clinically typical DS but apparently normal chromosomes. Her parents were consanguineous and she had a sister with a DS phenotype, who died at the age of 15 days. Repeated cytogenetic investigations (G-banding and high resolution banding) on the patient and her parents showed apparently normal chromosomes. Autoradiographs of quantitative Southern blots of DNAs from the patient, her parents, trisomy 21 patients, and normal controls were analyzed after hybridization with unique DNA sequences regionally mapped on chromosome 21. Sequences D21S59, D21S1, D21S11, D21S8, D21S17,D21S55, ERG, D21S15, D21S112, and COL6A1 were all found in two copies. Fluorescent in situ hybridization with a chromosome 21-specific genomic library showed no abnormalities and only two copies of chromosome 21 were detected. Nineteen markers from the critical region studied with polymerase chain reaction amplification of di- and tetranucleotide repeats did not indicate any partial trisomy 21. From this study we conclude that the patient does not have any partial submicroscopic trisomy for any segment of chromosome 21. It seems reasonable to assume that she suffers from an autosomal recessive disorder which is phenotypically indistinguishable from DS. © 1996 Wiley-Liss, Inc.

KEY WORDS: Down syndrome phenotype, chromosome 21

INTRODUCTION

Down syndrome (DS) is the most common cause of mental retardation, and is usually caused by the presence of an extra chromosome 21. Previous work has shown that the phenotype and the mental retardation of DS may be produced by duplication comprising only band 21q22. This was suggested in 1974 by Niebuhr and the critical chromosome segment was called the "Down syndrome region" [Epstein, 1986]. Several observations indicate that DS is a gene dosage disease. This means that overproduction of certain proteins encoded by normal genes on chromosome 21 disturbs the delicate balance of some biochemical pathways that are important for proper development and function of the organs affected in DS. The genes involved in the pathogenesis of DS are still unknown. Several reports on the molecular definition of the region of chromosome 21 that causes manifestations of the Down syndrome phenotype have been published [Delabar et al., 1987; Antonarakis et al., 1989; McCormick et al., 1989; Rahmani et al., 1989; Korenberg et al., 1990, 1992]. The molecular analyses suggest that severe mental retardation, the facial manifestations, and some of the other physical findings are associated with trisomy of the region 21q22.2–q22.3 including and distal to D21S55 [Rahmani et al., 1989; Korenberg et al., 1990, 1992; Delabar et al., 1993].

The aim of the present study was to analyze molecularly one patient with a phenotype of DS, but with apparently normal chromosomes, in an attempt to determine whether she carries any segment of chromosome 21 in triplicate or whether she has a DS phenotype due to another genetic abnormality.

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CLINICAL REPORT

The patient is a 37-year-old Caucasian woman. Her parents, II-4 and II-5 (see pedigree, Fig. 1), were half-sibs, and an autosomal recessive disorder was therefore suspected. The patient (III-6) was born after 36 weeks of gestation with a birth weight of 3000 g, when the mother was 21 years old and the father 26. She has one healthy half-sister (III-4) and a brother with no reported manifestations of DS (III-5), who died in the postnatal period due to prematurity (weight 1400 g). She had a younger sister (III-8), born after 38 weeks of gestation with a birth weight of 2300 g, who had, reportedly, a phenotype of DS, an unspecified heart defect, hypogammaglobulinemia, and thrombocytopenia, and died at the age of 14 days of bronchopneumonia (karyotype unknown). She also had a younger brother (III-9) (birth weight 3700 g) with no signs of DS except for bilateral simian creases. He was operated on for bilateral synblepharon, and suffered from immunodeficiency (dysgammaglobulinemia type 5A) and thrombocytopenia. He died of sepsis at the age of 12 years. The father died at the age of 58 of lung cancer. A son of her first cousin has DS with a regular trisomy 21 (IV-1). Photos or other documentation of clinical manifestations in the neonatal period concerning the proposita and her sister (III-8) are not available.

The proposita is profoundly mentally retarded (IQ 30). She is growth-retarded with a height of 1.27 m, and obese (weight 45 kg). She does not resemble the other relatives. At 33 years she had (Fig. 2) a flat face and broad, short neck with loose skin, brachycephaly, upwardly slanted palpebral fissures, slight divergent strabismus, flat nasal bridge, mild macroglossia, tooth crown shape abnormalities, and periodontal disease with absence of several teeth. She had a single transverse palmar crease on both hands and an excess of ulnar loops on fingertips (Fig. 3), clinodactyly of the 5th fingers, and an increased gap between the 1st and 2nd toe bilaterally. In Table I the clinical findings are listed following the established protocol of phenotype–genotype correlation [Epstein et al., 1991]. She had at least 12/20 criteria of DS according to Hall [1964], which is consistent with DS. (Present: premature or small for gestational age, upslanting palpebral fissures, brachycephaly, loose skin of neck, flat face and midface hypoplasia, short palate, mouth held open and protruding tongue, brachydactyly, simian crease, clinodactyly of 5th finger, wide space between first and second toes, abnormal teeth. Absent or unknown: hypotonia, abnor-

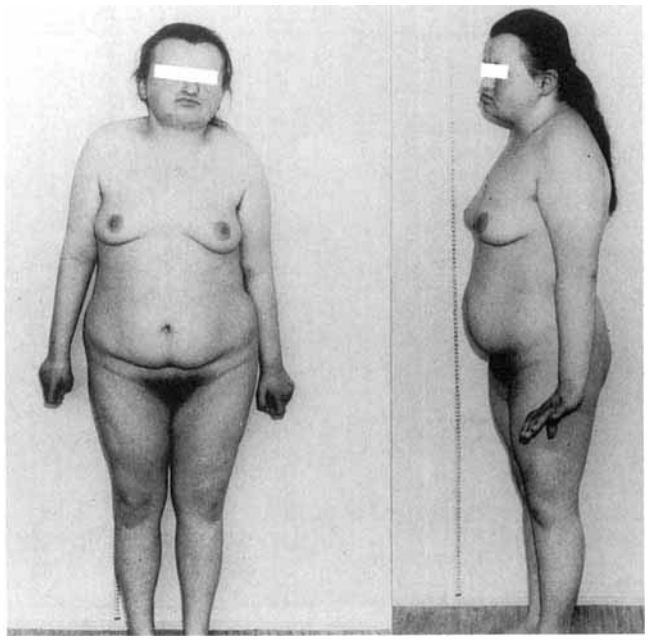

Fig. 2. The patient at the age of 20 years, with a DS phenotype.

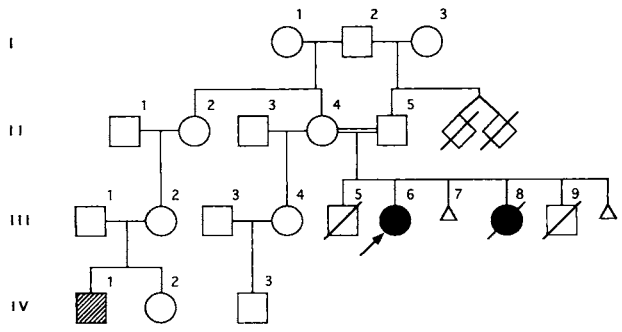

Fig. 1. Pedigree of the family. The proposita (III-6) is indicated by an arrow. Standard pedigree symbols are used with the filled circles representing a DS phenotype and the striped square representing verified regular trisomy 21.

Fig. 3. Palmprint of the patient. Note single transverse palmar crease, excess of ulnar loops on the fingertips, and clinodactyly of 5th finger.

TABLE I. Clinical Findings in the Proposita According to Protocol*

Measurements		Microdontia	No
Height	127 cm	Periodontal disease (several teeth lost)	Yes
Weight	45 kg	Ears	
Inner canthal distance	3.5 cm	Prominent upper helix	Yes
Palpebral fissure length	2.5 cm	Adherent lobuli	No
Ear length	5.5 cm	Helix root crossing concha	No
Chest circumference	82 cm	Narrow ear canals	Yes
Internipple distance	20 cm	Abnormal hearing test	No
Hand length	15 cm	Heart/gastrointestinal tract	
Palm length	9.5 cm	Congen. heart malform.	No
Tanner stage	5	Congen. GI tract malform.	No
Head		Celiac disease	Unknown
Brachycephaly	Yes	Obstipation	Yes
Flat face	Yes	Musculoskeletal	
Neck		Hip dislocation	No
Broad, short neck	Yes	Patellar subluxation	No
Loose skin of neck	Yes	Atlantoaxial/atlantooccipital instability	Unknown
Webbing	No	Hyperextensibility of joints	No
Eyes		Other: thoracic scoliosis	Yes
Upward slant of palpebral fissures	Yes	Skin and hair	
Epicanthal folds	No	Alopecia	No
Obstructed lacrimal ducts	No	Vitiligo	No
Blepharitis	No	Keratosis	No
Brushfield spots	Doubtful	Early graying	No
Peripheral iris hypoplasia	No	Other: male hairline	Yes
Nystagmus	No	Hands and Feet	
Strabismus	Yes	Brachyclinodactyly	Yes
Congen./acquir. cataract	No	Single fifth finger crease	Yes
Keratoconus	Unknown	Single transverse palmar crease	Yes
Myopia/hyperopia	Unknown	Sydney line	No
Nose		Increased gap 1st and 2nd toes	Yes
Flat nasal bridge	Yes	Webbing between 2nd and 3rd toes	No
Mouth		Other	
Down-turned corners	Yes	Thyroid disorders	Unknown
Prominent lower lip	Yes	Diabetes	No
Protruding tongue	Yes	Psychiatric disorder	No
Furrowed tongue	Yes	Behavior disorder	No
Narrow, short palate	Yes	Seizures	No
Cleft uvula/palate	No	Leukemia	No
Submucous cleft	No	Other: frequent infections during the first 3 years	Yes
Teeth			
Malformed teeth	Yes		

*Epstein et al. [1991].

mal Moro reflex, epicanthic folds, Brushfield spots, low-set ears, snuffling breathing, hypermobile joints, abnormal acetabulum.)

MATERIALS AND METHODS

Cytogenetics

G-banded and prophase banded chromosomes were studied in cells from skin (twice) and blood (three times) from the patient, and over all more than 800 cells were analyzed. G-banded and prophase banded chromosomes of the parents and the sibs III-4 and III-9 were analyzed in 200 cells/person.

Molecular Analysis

The DNA probes used were D21S59, D21S1, D21S11, D21S8, D21S17, D21S55, ERG, D21S15, D21S112, and COL6A1 specific for chromosome 21 (Fig. 4), and Met H and B 79A specific for chromosome 7. See Table II for information concerning the restriction enzymes used, number of alleles, sizes of fragments, and depositors. Probes were 32-P labeled by random priming (Multiprime 1600Z, Amersham) to a specific activity of 10^8 cpm/μg.

DNA from healthy individuals, patients with regular trisomy 21, and the patient and her parents was prepared from blood by standard techniques. The DNA was digested with different restriction enzymes, size separated by agarose gel electrophoresis, and blotted onto nylon membranes (Hybond, Amersham) [Sambrook et al., 1989]. The membranes were hybridized to the different probes and the autoradiographs obtained were scanned on a Shimadzu CS-930 scanner. The copy number of each probe was determined by densitometric comparison of the intensity of the allelic bands. When the patient was homozygous at a locus, hybridization with a probe specific for chromosome 7 was carried out simultaneously. The intensity of the bands corresponding to the different chromosomes was compared, and the copy number was determined in relation to the control DNA from healthy and trisomy 21 individuals. For the VNTR markers used, the number of alleles could be correlated to the copy number of the segment.

The PCR amplified microsatellite markers D21S11, D21S16, D21S120, D21S145, D21S167, D21S168, D21S171, D21S172, D21S189, D21S210, D21S212,

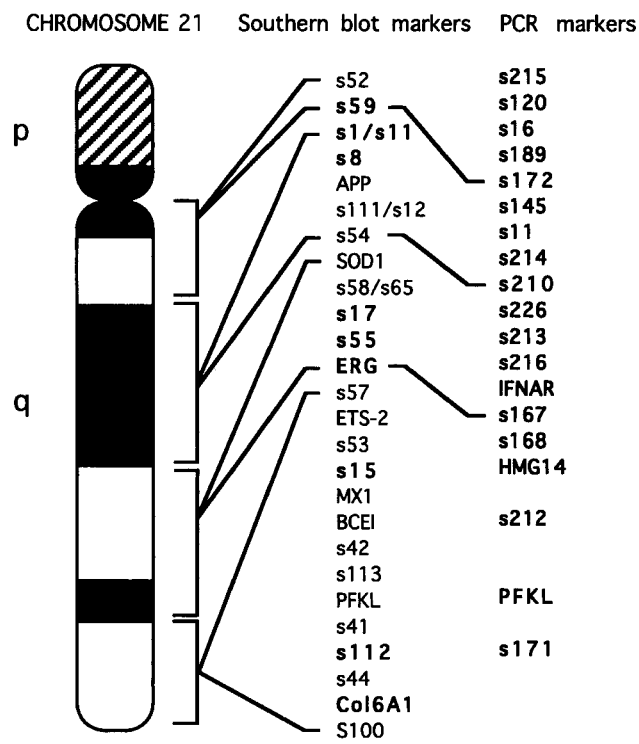

Fig. 4. Chromosome 21 with the approximate locations of some chromosome-21 specific markers. The markers analyzed in the family are in bold.

D21S213, D21S214, D21S215, D21S216, D21S226, HMG14, IFNAR, and PFKL (Fig. 4) [Van Camp et al., 1991, McInnis et al., 1993] located on chromosome 21 were used in densitometric studies [Petersen et al., 1991]. The PCR reactions were performed in 10 μl, and with one 32-P end-labeled primer (standard procedure), as follows: 1× (95° 4′, 55° 30″, 72° 30″), 26× (95° 30″, 55° 30″, 72° 30″). The primer concentration was 1 μM, and 10 ng template DNA was used for a 10 μl reaction. The PCR products were separated on a 6% denaturing polyacrylamide gel which was then fixed, dried, and exposed to X-ray film for 12 to 24 hr. The intensity of the allelic bands was compared as described for the Southern blot markers.

In Situ Hybridization

A chromosome 21-specific genomic library (ATCC 57743) was biotinylated by nick translation and hybridized to metaphase and interphase spreads of the patient's and her mother's chromosomes. For detection, FITC-conjugated antibiotin antibodies were used [Lichter et al., 1988]. The slides were analyzed using a confocal laser scanning microscope (Leica).

RESULTS

Cytogenetics

Normal chromosomes were found in the patient, her parents and the two sibs III-4 and III-9 after G and prophase banding.

Molecular Analysis

The patient has the segments D21S59, D21S1, D21S11, D21S8, D21S17, D21S55, D21S15, and ERG in two copies according to densitometric dosage studies (exemplified by marker D21S11 in Fig. 5). At the VNTR polymorphism in locus D21S112 the patient has 2 alleles. Her parents are heterozygous and she has inherited a distinct allele from each parent. Previously reported results [Annerén et al., 1991; Annerén and Edman, 1993] indicated that the patient had three alleles at the VNTR polymorphism in the COL6A1 gene. However, because of unclear information at an early stage regarding the allelic pattern at the COL6A1 locus, we initially misinterpreted our results concerning this VNTR marker. The patient has in fact inherited different alleles, one from each parent, at the COL6A1 locus and thus carries this segment in two copies only.

With the microsatellite markers used, no duplication was detected (exemplified by marker PFKL in Fig. 6). The patient was homozygous for only 5 of the 19 PCR markers analyzed (Table III).

TABLE II. Probes and Restriction Enzymes Used for Southern Blotting: The Number of Alleles and the Sizes of Fragments Are Given, as Well as Depositors

Probe	Enzyme	Alleles (size in kb)	Depositor
D21S59	*Taq*I	2 (A1: 2.7, A2: 2.0)	P. Watkins
D21S1[a]	*Bam*HI	2 (A1: 7.0, A2: 6.3)	P. Watkins
D21S11[a]	*Eco*RI	2 (A1: 1.9, A2: 2.9)	P. Watkins
D21S8[a]	*Hind*III	2 (A1: 2.7, A2: 3.2)	P. Watkins
D21S17	*Bg*III	2 (A1: 18.5, 0.57, A2: 12.3, 0.43)	M.A. Ferguson-Smith
D21S55[a]	*Xba*I	6 (2.2–2.6)	P. Watkins
ERG[a]	*Bg*II		N. Sacchi
D21S15[a]	*Msp*I	2 (A1: 4.1, A2: 3.4, 0.7)	M.A. Ferguson-Smith
D21S112	*Rsa*I	Multiple	Collaborative Research Inc.
COL6A1	*Taq*I	6 (4.2–5.2)	Mon-Li Chu
MetH	—	—	P. O'Connell
B79A	—	—	B. Wainwright

[a] Polymorphism not informative in the family investigated; simultaneous hybridization with a probe specific for chromosome 7 (MetH or B79A) was carried out for copy number analysis.

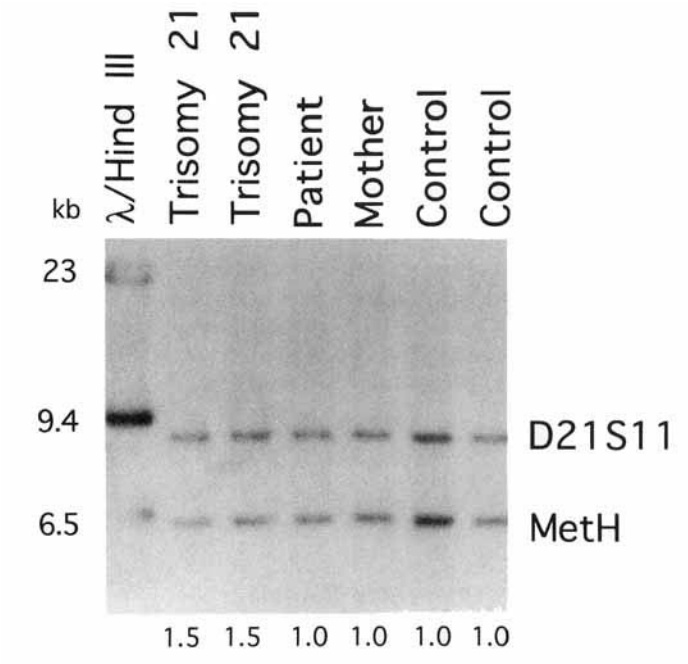

Fig. 5. Autoradiogram showing Southern blot analysis of genomic DNA digested with *Bgl*II and hybridized with a probe from locus D21S11 and the marker MetH specific for chromosome 7. The intensity of the bands is quantitated by densitometry. As indicated below each lane, the ratio (D21S11/MetH) of the peaks is 1.5 for both of the trisomy 21 controls to the left, and 1.0 for the normal controls to the right. The patient and her mother both have the ratio 1.0, indicating two copies of this chromosome 21 segment.

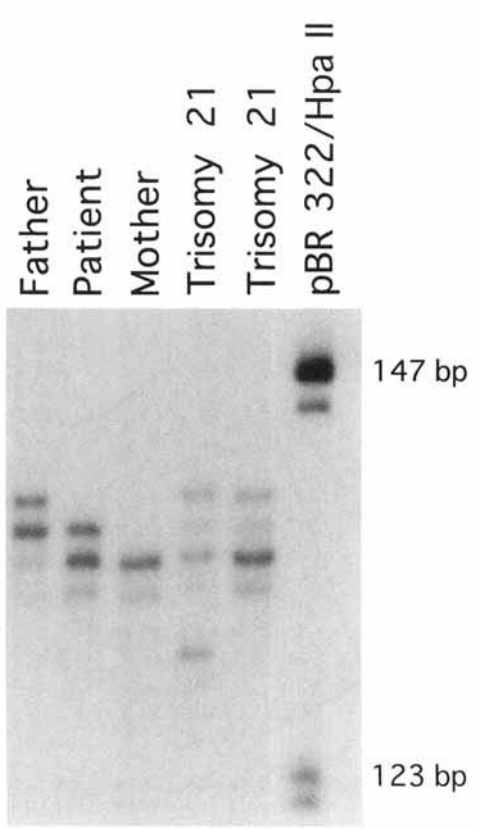

Fig. 6. Autoradiogram showing PCR analysis of the microsatellite marker PFKL. The father has alleles 1 and 2, the patient alleles 2 and 3 (each in one copy only), and the mother is homozygous for allele 3. The two following lanes show trisomic controls, one having three different alleles, 1, 3, and 6, and the other having one copy of allele 1 and two copies of allele 3. The last lane contains a size marker, pBR 322/*Hpa*II.

In Situ Hybridization

With the chromosome 21-specific genomic library used, no evidence was found for translocation of material from chromosome 21 in the patient or her mother.

DISCUSSION

Since Niebuhr [1974] reported on a patient with a DS phenotype and a partial trisomy 21, several similar cases have been described [Poissonnier et al., 1976; Hagemeijer and Smit, 1977; Delabar et al., 1987; Rahmani et al., 1989; Korenberg et al., 1990]. Great efforts have been made to define the region of chromosome 21 whose trisomy results in DS [Delabar et al., 1987; Antonarakis et al., 1989; McCormick et al., 1989; Rahmani et al., 1989; Korenberg et al., 1992; Delabar et al., 1993]. In order to do this, a physical map of chromosome 21 has been established and several markers have been defined [Watkins et al., 1987; Antonarakis et al., 1989; Burmeister et al., 1991; Creté et al.,1993]. The "DS region" is hitherto defined as band 21q22.2 and parts of band 21q22.3, including marker D21S55 [Rahmani et al., 1989; Korenberg et al., 1990; Delabar et al., 1993]. Attempts have been made to map molecularly the different phenotypical abnormalities of DS, such as the congenital heart defect and duodenal stenosis [Korenberg et al., 1992]. The ultimate goal is to find the genes responsible for the DS phenotype. Since there are very few patients with partial trisomy 21 and DS, an international working group (NICHD) was established and a protocol for genotype–phenotype correlations was constructed in order to facilitate the process [Epstein et al., 1991].

We now describe a 37-year-old woman with profound mental and growth retardation and with a phenotype of DS, but with microscopically normal chromosomes. None of 19 polymorphic PCR markers (microsatellite markers) gave a pattern indicating trisomy for any part of chromosome 21. Owing to misleading information on the allelic pattern of the marker at the COL6A1 locus, we misinterpreted our initial results and reported three alleles at the COL6A1 gene [Annerén et al., 1991; Annerén and Edman, 1993]. We have studied the allelic pattern of COL6A1 extensively and we now know that the patient has inherited two different alleles, one from each parent. Using quantitative Southern blotting and VNTR polymorphisms, polymorphic PCR markers and chromosome 21-specific fluorescent in situ hybridization, we have now excluded any duplication of the chromosome. As can be seen in Table III, the patient is homozygous at only 5 of the 19 microsatellite marker loci analyzed. Since these 5 markers are dispersed on the chromosome, we can conclude that she has not inher-

TABLE III. Chromosome 21 PCR Polymorphisms: Genotypes of the Patient and Her Parents

Locus	Father	Child	Mother
D21S215	1,2	2,2	2,3
D21S120	1,2	2,3	3,1
D21S16	2,3	3,1	1,3
D21S189	1,3	3,2	2,3
D21S172	1,1	1,2	2,1
D21S145	1,2	2,1	1,1
D21S11	3,2	2,1	1,4
D21S214	1,1	1,2	2,2
D21S210	2,3	3,1	1,3
D21S226	1,1	1,1	1,1
D21S213	1,1	1,1	1,1
D21S216	2,1	1,2	2,2
IFNAR	2,1	1,1	1,3
D21S167	2,2	2,1	1,2
D21S168	1,2	1,2	1,2
HMG14	2,1	1,1	1,1
D21S212	2,3	3,1	1,1
PFKL	1,2	2,3	3,3
D21S171	1,3	3,2	2,3

ited large continuous parts of chromosome 21 that her closely related parents possibly could have in common.

Several patients with mental retardation and DS phenotype but without any molecularly detectable anomalies have been reported previously [Delabar et al., 1987; McCormick et al., 1989]. We have performed molecular analyses of 5 similar patients and have been able to exclude trisomy of any part of chromosome 21 (unpublished data). It is reasonable to assume that there exists a disorder with mental retardation and a DS phenotype whose cause is not trisomy of any part of chromosome 21.

One possible but not very likely explanation of the disorder in the present proposita is a defect in the regulation of transcription or translation of certain critical genes on chromosome 21, leading to an increased amount of the corresponding proteins as seen in patients with trisomy 21. More likely, since the parents were half-sibs, and since a sister of the proposita seems to have suffered from the same disorder as the proposita, the phenotype of the proposita might have been caused by an autosomal recessive gene. If so, this could be the first time that a monogenic disorder has been found to result in a phenotype that cannot be distinguished from DS.

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